# Engineered Ribozymes as Molecular Tools for Site-Specific Alteration of RNA Sequence

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Over the past two decades our understanding of RNA has been revolutionized. Ribozymes that catalyze phosphoryl transfer reactions have been discovered in nature[1] and are being joined by artificial ribozymes possessing activity for catalysis of an increasing number of chemical reactions.[2] The spliceosome and the ribosome are organized as ribonucleoprotein particles of enormous size, with the RNA part essential to function.[3] The nontranslated leader region of mRNAs can directly bind small molecules that are often the final product of a metabolic reaction catalyzed by the encoded protein, and in this manner the leader acts as a riboswitch for genetic regulation.[4] Based on the capacity of RNA to bind molecules with high specificity, a number of 'aptazymes' have been developed that transform the binding event at the aptamer domain into a signal for switching activity of a ribozymic component contained in the same molecule.[5]

With these exciting discoveries, RNA has also become the focus of investigations into novel therapeutic schemes. *Trans*-cleaving ribozymes,[6] antisense RNAs,[7] and short interfering RNAs (siRNAs)[6c, 8] have been used to silence undesired gene expression. All three strategies have in common the fact that the synthesis of a protein that is pathogenic to the cell is inhibited at the level of the messenger RNA. Thus, antisense RNAs as well as *trans*-cleaving ribozymes are of considerable use for treatment of malignant or viral diseases. However, if one considers the therapy of inherited diseases such as cycstic fibrosis or sickle cell anaemia, strategies that allow correction of genetic disorders rather than destruction of the faulty transcript are required. 'Repairing ribozymes' may be uniquely suited for this purpose because, compared to more traditional methods that attempt to correct a genetic deficiency by transferring a wild-type DNA version of a gene to the cell, ribozymes might repair incorrect transcripts without interfering with the corresponding DNA.

Over the past decade, four major approaches have emerged to modulate gene function at the RNA level:[9] *trans*-splicing by group I intron ribozymes,[9a] *trans*-splicing mediated by the spliceosome,[9b] use of antisense oligonucleotides to modify splicing pathways,[9c] and antisense oligonucleotides that are capable of activating the cellular enzyme double-stranded RNA adenosine deaminase (ADA).[9d] Work in my laboratory has focused on the development of twin ribozymes for site-specific alteration of RNA sequence.[10] These twin ribozymes have not yet been tested with genetically relevant RNA sequences in cell culture systems. However, their capacity to efficiently alter the sequence of a given RNA in vitro has been successfully demonstrated.[10b] In addition to the ribozyme- or antisense-oligonucleotide-based strategies mentioned above, a number of other schemes have been investigated to demonstrate the potential of nucleic acid repair in functional genomics. For example, mobile group II introns, catalytic RNA structures capable of inserting themselves directly into a chromosome and subsequently being reverse transcribed into DNA, have been successfully used to target DNA sites in human cells.[11] Single-stranded triplex-forming oligonucleotides have been applied to correct a mutation in plasmid DNA[12] as well as an ADA mutation in human lymphocytes by facilitating DNA repair of the mutation through the nucleotide excision repair pathway.[13] Furthermore, double-stranded oligonucleotides capped by hairpins and consisting of both DNA and RNA residues (so-called chimeras)[14] have shown efficacy in correcting single-base genomic changes in mammalian,[15] animal,[16] and plant cells.[17]

When the strategies for induced repair at the RNA and at the DNA level are compared, it becomes clear that there is an important advantage on the RNA side. Any processing at the DNA level may interfere with gene expression and, in the worst case, produce unintended effects, such as disruption of important genes through insertional mutagenesis. If a gene is involved that normally regulates cell growth and division, cancer may be the result. The recently reported cases of two French gene therapy patients with severe combined immune deficiency (SCID) who developed leukaemia after treatment with the correct version of the defective gene[18] dramatically prove that this problem must be seriously considered. In these trials, the corrective genes were packaged into modified retroviruses, which can incorporate themselves into the DNA of a host cell. The retroviruses were stripped of most of their viral genes. However, it is not possible to control where retroviral vectors insert themselves. It seems that in the reported cases, the corrective gene integrated itself into or near to a gene that can cause childhood leukaemia.[18] Thus, even if the treatment works well, the risk is not acceptable. Against this background, alternative strategies that correct genetic disorders while leaving the naturally regulated gene expression intact are urgently required.

Among the strategies mentioned above for sequence alteration at the RNA level, only *trans*-splicing group I intron ribozymes mediate the repair process by themselves.[9a] Spliceo-

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some-mediated *trans*-splicing,[9b] as well as the use of antisense oligonucleotides for modification of splicing pathways[9c] or activation of adenosine deaminase,[9d] requires the help of cellular enzymes to correct a mutated RNA sequence. Strikingly, while antisense oligonucleotides are responsible for recognition of the mutated sequence, cellular proteins are the major players in the processing of the target RNA. Ribozymes, on the contrary, combine both recognition elements and functionality and thus allow precise engineering to mediate the repair reaction in the most successful way. Herein, strategies applying group I intron ribozymes[9a] and an approach based on recently developed twin ribozymes[10b] are discussed.

## *Trans* Splicing by Group I Intron Ribozymes

Introns are noncoding sequences that interrupt the coding sequences (exons) of most eukaryotic genes. To ensure the expression of functional mRNAs, rRNAs, and tRNAs, intron sequences must be removed after transcription to give the mature RNA molecules (splicing, Figure 1). For group I introns, this process is mediated by the autocatalytic activity of the intron RNA itself. The first evidence of self splicing came from work on ribosomal RNA genes of *Tetrahymena thermophila*. Although in vivo the splicing reaction occurs with the assistance of protein factors, efficient RNA self splicing has been demonstrated in vitro.[19] The splicing process involves a two-step transesterification and is dependent on the presence of divalent metal cations, typically $Mg^{2+}$ ions, and a guanosine cofactor. In the first step, nucleophilic attack by the 3′-OH group of the guanosine cofactor on the phosphorus atom in the phosphodiester bond at the 5′-splice site results in the cleavage of the 5′-exon, which results in it carrying a free 3′-hydroxy group. This group attacks the phosphorous atom in the phosphodiester bond at the 3′-splice site in the second step, which leads to the spliced RNA product by ligation of the 5′-exon with the 3′-exon and release of the intron (Figure 1).

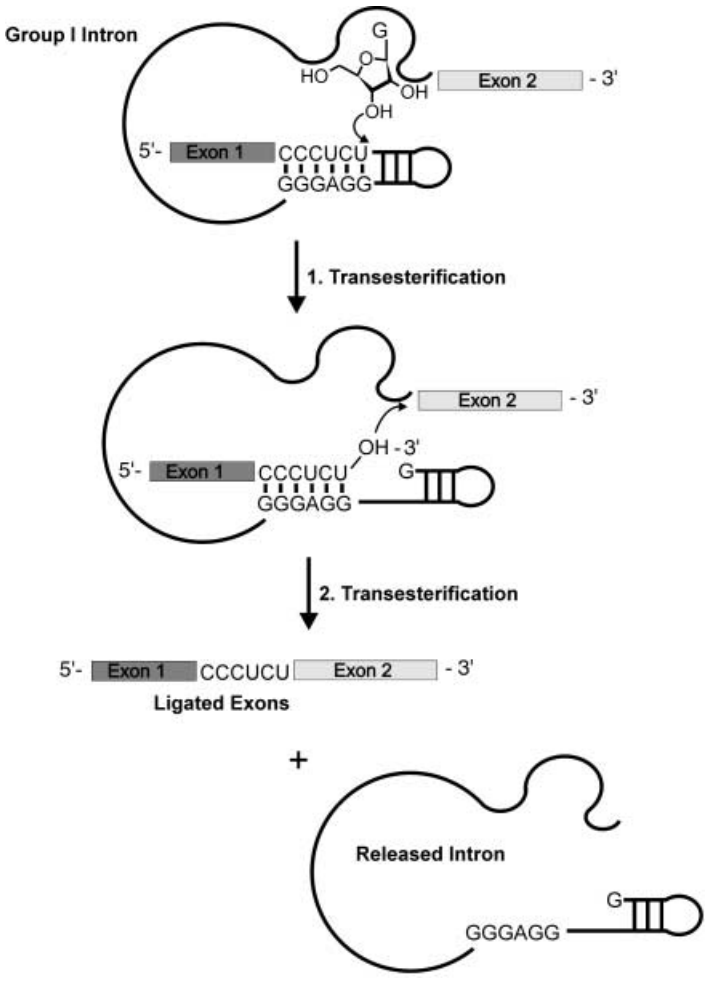



***Figure 1.*** *Self splicing of group I introns. The guanosine cofactor is bound to the intron and initiates splicing by nucleophilic attack at the 5′-splice site. As a result of a two-step transesterification, the 5′-exon is ligated to the 3′-exon and the intron is spliced out.*

The *Tetrahymena* group I intron can be integrated into other systems to mediate self splicing. For example, it has been shown to efficiently splice itself from *lacZ* transcripts in *Escherichia coli* to generate a functional mRNA for translation of the $\alpha$-complement of $\beta$-galactosidase.[20] Sullenger and Cech tested whether the *Tetrahymena* intron could perform a similar reaction in *trans*.[9a] To this end, they designed a model system that consists of the two molecules shown in Figure 2. One RNA, the 5′-exon (*5′lac*), contains the first 31 nucleotides of the *lacZ* transcript and the recognition sequence CCCUCU followed by two adenosine residues. The second RNA, termed the ribozyme *3′lac* RNA, consists of an engineered ribozyme RNA (derived from the *Tetrahymena* intron[21]) covalently linked to a 3′-exon that encodes 67 amino acids of the $\alpha$-complement of $\beta$-galactosidase. The ribozyme *3′lac* RNA binds to *5′lac* through its complementary 5′-terminating sequence GGAGGG. *Trans* splicing then involves cleavage of the two 3′-adenosine residues followed by ligation of the resulting 5′-product to the *lacZ* 3′-exon. The net result of these reactions is a transcript containing the correct translational reading frame for $\beta$-galactosidase and the released ribozyme (Figure 2). In a number of experiments, Sullenger and Cech convincingly demonstrated that the ribozyme accurately functions in vitro as well as in *E. coli*.[9a]

Group I intron ribozymes recognize their splice sites through the internal guide sequence (IGS) and complementary base pairing to their 5′-exon binding site; the only specific requirement is the presence of a uridine residue 5′ to the cleavage site that forms a wobble pair with a conserved guanosine residue in the IGS (see Figure 1).[22] This flexibilty allows adaptation of targeted *trans* splicing to correct a variety of mutant transcripts by following the strategy outlined in Figure 3. Since the groundbreaking results of Sullenger and Cech, a number of studies have demonstrated that *trans* splicing is a powerful tool for potential therapeutic application.[23] Since the successful demonstration of *trans* splicing in *E. coli*,[9a] truncated *lacZ* transcripts have been corrected in mammalian cells too.[23a, 24] Group I intron *trans* splicing has been utilized to correct sickle-cell transcripts[23b,c] and *trans*-splicing ribozymes have been designed for correction of a triplet repeat expansion in the 3′-untranslated region of the myotonic dystrophy protein kinase transcript,[23d] as well as to repair mutant p53 transcripts[23e] and the mRNA of a mutant

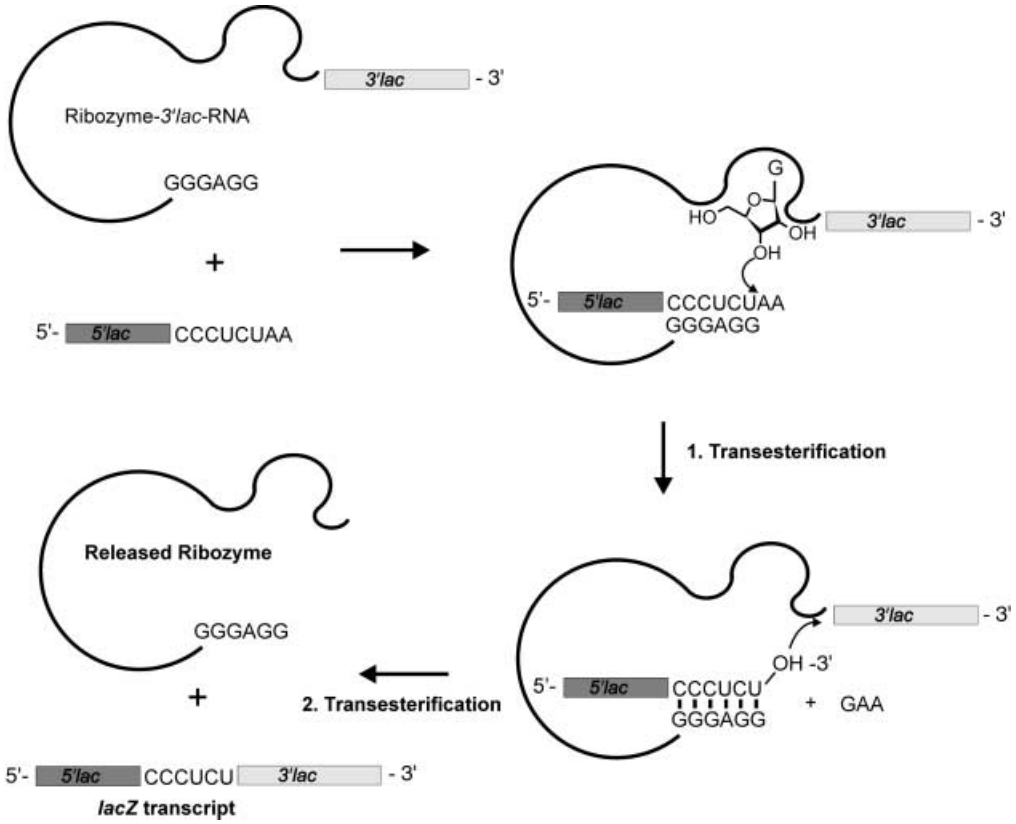



***Figure 2.*** *Mechanism of* trans *splicing of* lacZ *transcripts by a ribozyme derived from a group I intron, as suggested by Sullenger and Cech.[9a] The* trans*-splicing ribozyme is covalently linked to the 3′-exon (*3′-lac*) and contains the six-base sequence GGGAGG required for recognition of the corresponding 5′-exon (*5′lac*). The splicing reaction leads to ligation of the 5′-exon to the 3′-exon and thus generates a functional* lacZ *transcript and a released ribozyme.*

canine skeletal muscle chloride channel.[23f] In a very recently reported study, *trans*-splicing ribozymes were used to convert pathogenic transcripts of the hepatitis C virus (HCV) into new RNAs that exert anti-HCV activity.[25] While *trans* splicing was found to occur with a half time of 13 min and 85 – 90% conversion of precursor into *trans*-spliced RNA in vitro,[9a] the reaction initially processed "only" 25 – 50% of targeted transcripts in vivo.[24a] This difficulty is likely to result to a considerable extent from the relatively short 5′-exon binding site (IGS) of group I introns (only six nucleotides) limiting the sequence specificity of the ribozyme for its target. Indeed, it has been observed that many nontargeted transcripts were modified in addition to the target RNA.[23a] This problem could be partially overcome by structural optimization of the *trans*-splicing ribozymes.[26] In particular, extension of the IGS helped to increase the affinity and specificity of the ribozyme for its target RNA.[27]

### RNA Sequence Alteration by Twin Ribozymes

My research group has recently introduced an alternative scheme for revising RNA messages.[10b] This scheme relies on a small engineered twin ribozyme and a small RNA repair oligonucleotide to site-specifically alter a given RNA sequence. *Trans* splicing by group I intron ribozymes as described above requires a large processing ribozyme fused to the reconstituting nucleotide segment, plus the entire mRNA sequence downstream of the mutation. For example, *trans* splicing of *lacZ* transcripts encoding the $\alpha$-complement of $\beta$-galactosidase in *E. coli* required a 416-nucleotide ribozyme followed by 200 nucleotides of the *lacZ* sequence.[9a] Most transcripts of functional genes are longer than that and, dependending on the location of the mutation (5′- versus 3′-side), many transcripts of medical relevance require a very large reconstituting nucleotide segment to be delivered, which makes the ribozyme – 3′-exon fusion construct even longer than 616 nucleotides.

To limit the size of the RNA processing ribozyme, we sought for a general method of manipulating at will any chosen patch of any given mRNA sequence. While point mutations could be repaired by base replacement strategies, the development of a methodology allowing the exchange of entire RNA segments seems more reasonable since a wide range of mutations could then be targeted with the same machinery, independent of their particular nature. Such a fragment exchange strategy requires an agent which promotes, in a strictly controlled fashion, two chain cleavage events and two ligations. We have engineered a small RNA consisting of 141 nucleotides that is derived from the hairpin ribozyme by tandem duplication (hence dubbed 'twin ribozyme') and meets all the functional requirements of such an approach[10b] (Scheme 1). The hairpin ribozyme is a catalytic RNA structure occurring in nature.[28] This ribozyme catalyzes the reversible site-specific cleavage of RNA substrates to generate fragments with a 2′,3′-cyclic phosphate and a free 5′-OH terminus, respectively. In turn, the reverse reaction requires fragments with the same characteristic termini to be ligated.

The specific structure of the ribozyme – substrate complex determines the extent of cleavage versus ligation. RNA fragments that are stably bound to the ribozyme are preferentially ligated, whereas fragments that are less well bound (but stable enough to allow folding into a catalytically competent structure) favor cleavage.[29] By considering the characteristic cleavage/ligation properties of the hairpin ribozyme, the twin ribozyme

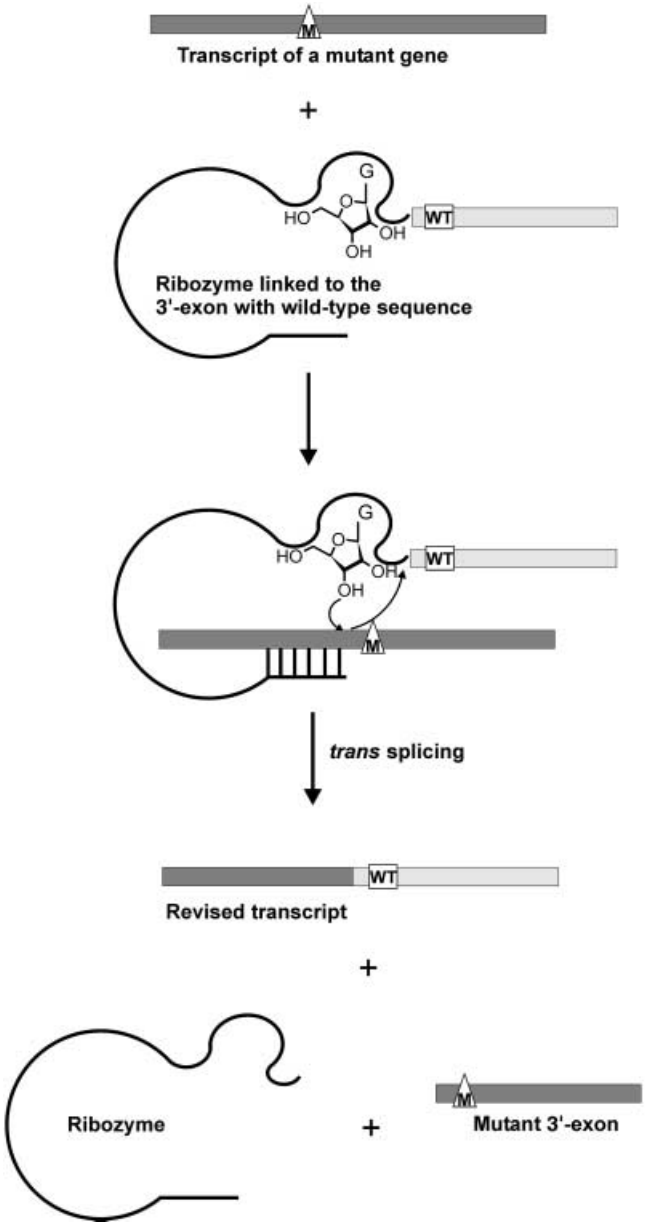


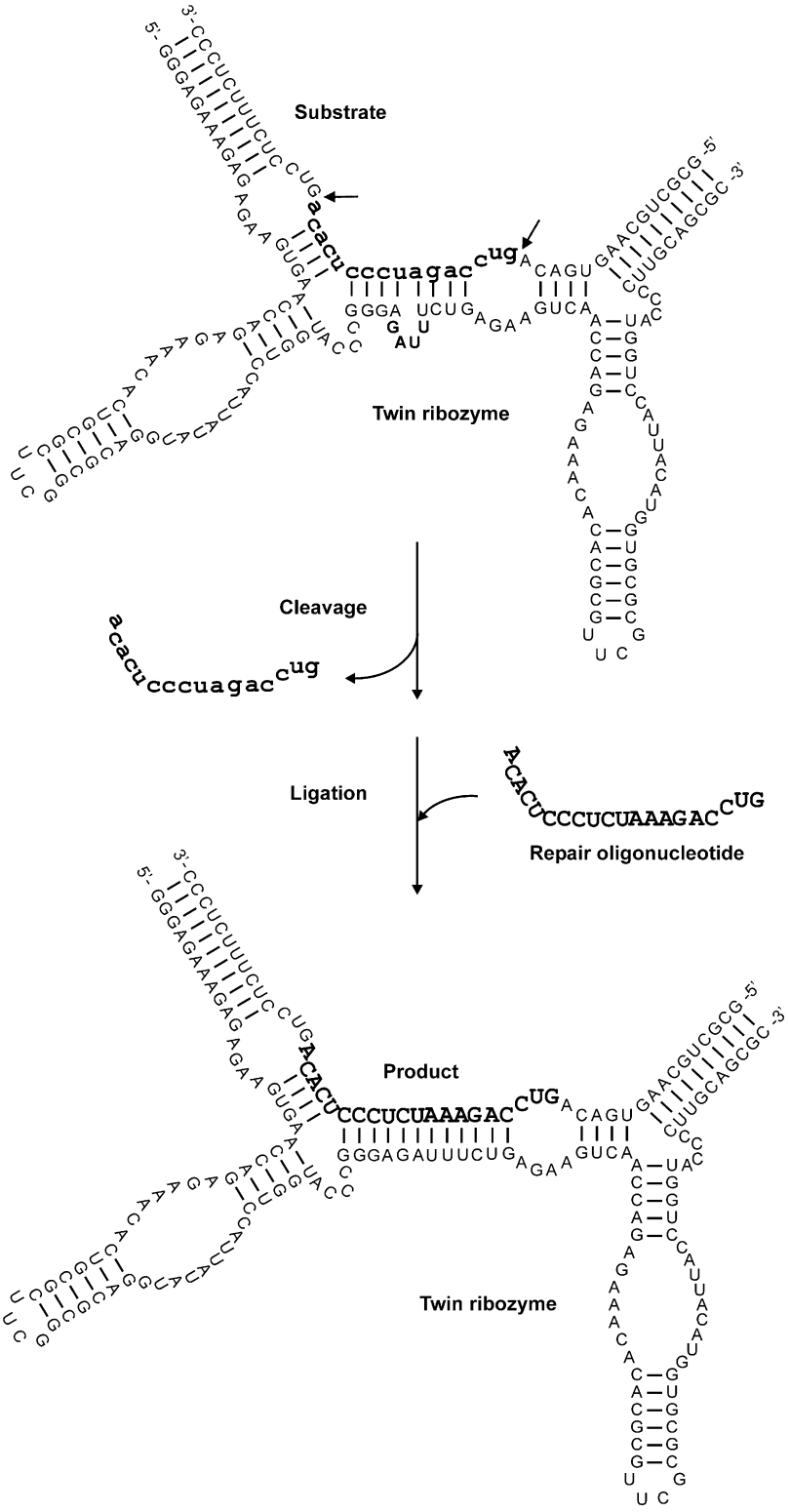

***Figure 3.*** *Targeted* trans *splicing for correction of genetic disorders at RNA level. M, mutation; WT=, wild-type sequence.*

shown in Scheme was designed to insert four nucleotides into a predetermined site of an arbitrary model RNA, and thus to mimick the correction of a deletion mutation at the RNA level. In the ribozyme–substrate complex, a stretch of four nucleotides in the central part of the ribozyme strand (GAUU), is looped out. Cleavage at both predetermined sites leads to a 16-mer that can easily dissociate from the ribozyme as a result of the loop weakening its binding. In contrast, the two RNA fragments flanking the cleavage sites form contiguous duplexes with the ribozyme of 11 and 14 base pairs, respectively, and thus should preferentially remain bound. The added 'repair oligonucleotide' contains the four additional nucleotides complementary to the GAUU loop in the ribozyme strand. Hence, binding of this oligonucleotide to the gap left by dissociation of the 16-mer extends the formerly interrupted double-stranded stretch by four nucleotides and makes it contiguous to the ribozyme sequence and likely to be preferentially ligated. As a result, 16 nucleotides of substrate sequence are exchanged for 20 nucleotides that were separately added as a synthetic repair oligonucleotide. In initial experiments, up to 30% of the input substrate was converted into insertion product;[10b] more recent experiments under varied conditions delivered the product with up to 50% yield.[10c]

In all these studies, the sequences bordering the deletion as well as the deletion itself were defined arbitrarily and thus the question of whether this strategy can be used with genetically relevant RNA sequences remains. The basic hairpin ribozyme can be adapted to process different RNA sequences with remarkable flexibility and specificity.[30] This possibility, along with the application of evolutionary methodologies such as selection in vitro and in vivo should pave the way for the design of twin ribozymes addressing RNAs with sequence failures of medical relevance. In analogy to the model experiment, diseases that are caused by short deletions (such as cystic fibrosis[31]) are the most obvious first targets. However, the approach should also be suitable for the correction of replacements and insertions. In these cases the repair reaction could be driven by the same strategy as is used in the deletion mutation model. Mismatches between input substrate and ribozyme (in the case of replacement mutations) or inserted nucleotides within the input substrate looped out upon binding to the ribozyme (in the case of insertion mutations) should destabilize the mutated fragment so that it can easily be released after cleavage. The repair oligonucleotide can in both cases be designed to form a stable contiguous duplex with the ribozyme to be ligated to the remaining fragments of the input RNA. Additionally, a larger excess of the repair oligonucleotide (a 2.5-fold excess was used in the experiment described above) could help drive the reaction.

Compared to RNA repair by *trans*-splicing ribozymes, the twin ribozyme approach is still in its infancy. Twin-ribozyme-mediated RNA repair has not yet been tested in vivo. Furthermore, as mentioned above, the initial experiments have been carried out with arbitrarily chosen substrates. Twin ribozymes that target a variety of different sequences need to be developed in order to demonstrate the potential of this strategy for universal application. Further structural optimization and improved functional design should help to increase the so far obtained 50% yield of conversion of input substrate into product. Nevertheless, the twin ribozyme strategy is a promising alternative to *trans*-splicing ribozymes in terms of applicability and specificity and thus could be used for editing genetic disorders at the RNA level, as illustrated in Figure 4. The key advantage lies in the fact that sequence alteration is achieved in a patchwise fashion and hence any accessible target sequence, irrespective of its location within the relevant mRNA, can be addressed with the same simple machinery.




***Scheme 1.*** *Twin-ribozyme-mediated RNA sequence alteration. Substrate RNA is annealed to the twin ribozyme (top) and cleaved at two defined sites (indicated by arrows). The fragment extending between the two cleavage points (16-mer, lower-case letters, bold) is replaced on the ribozyme by the repair oligonucleotide (20-mer, bold capital letters), which is subsequently ligated to the flanking substrate fragments to form the twin ribozyme – product complex (bottom). For details, see the main text.*

## Summary and Future Prospects

Nature has evolved elegant ways of initiating, maintaining, and regulating life. RNA molecules fulfil a number of essential biological functions. The investigation and understanding of these functions has in turn allowed the creation of RNA molecules in the laboratory with improved or even altered function. Among a wide variety of potential applications of these functional RNA molecules exciting developments have emerged in the field of RNA therapeutics. Beyond doubt, one of the most exciting recent discoveries is the finding that interfering RNA (RNAi) can be utilized to silence gene expression in mammalian cells, which gives tremendous hope for new classes of therapeutics to treat cancer, viral infections, and other diseases. However, an additional option is to use engineered ribozymes to site-specifically alter the sequence of a deleterious mRNA from a dominant mutant gene or a virus. The leading methodology in this area is *trans* splicing by engineered group I intron ribozymes, which in future might be accompanied by the twin ribozyme approach described above. It should however be noted that it remains to be seen if RNA revision can indeed be useful in the clinic for the treatment of genetic deficiencies. While twin ribozymes have not yet even been tested in living cells, *trans*-splicing ribozymes can process only up to 50% of the targeted transcripts.[24a] This must not necessarily be a serious drawback; in certain diseases the correction of only a fraction of the mutant transcripts might have a clinical benefit. Nevertheless, enhancing the yield of functional product is one of the major goals in the development of the next generation of *trans*-splicing ribozymes and twin ribozymes. A hurdle to be overcome in both strategies is the development of effective delivery systems capable of targeting specific cell types. Even if useful repair ribozymes can be made, improvement in cell delivery and expression systems will be required to give stable enough expression of ribozymes inside cells to allow them to repair the mutant RNA for extended periods of time. This problem is common to all areas of gene therapy and there is reason to be optimistic that efficient gene delivery approaches will be forthcoming in future.

Overall, the concept of direct RNA revision is intriguing and the application of engineered repairing ribozymes in gene therapy seems feasible. In contrast to most standard gene therapy approaches, which can cause mutations through integration into the genome of the

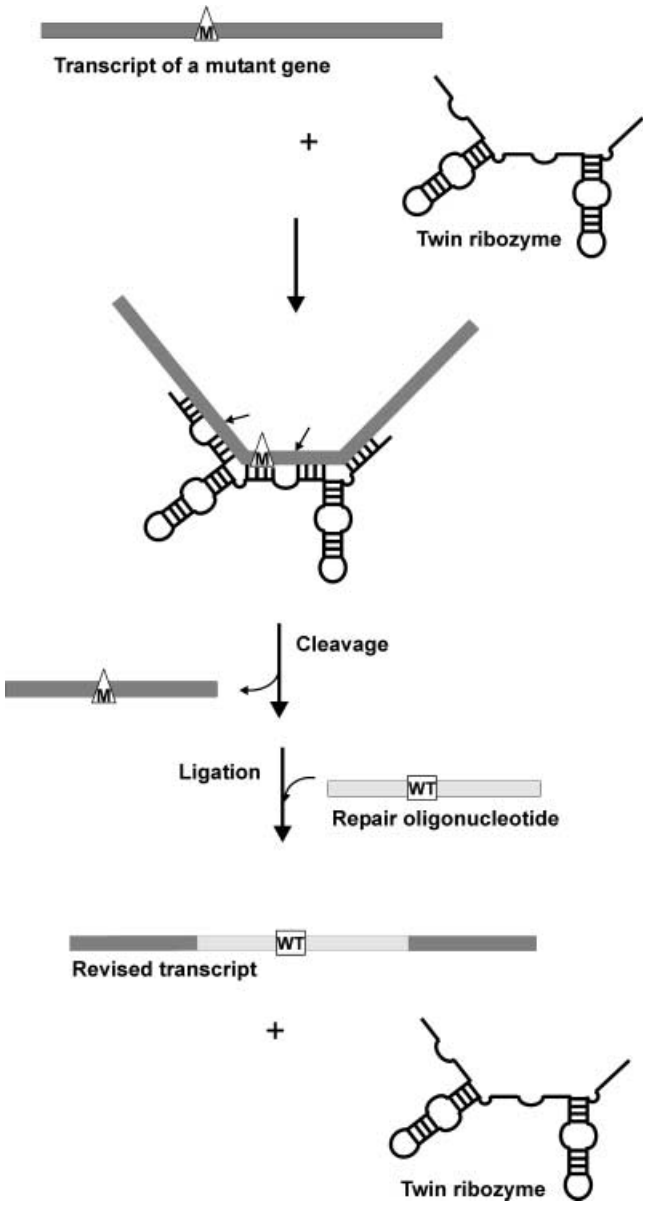



***Figure 4.*** *Suggested scheme for using twin ribozymes as therapeutic tools for sequence correction of transcripts carrying genetic disorders. The number of schematically shown base pairs does not correspond with the real number of base pairs in the ribozyme or between the ribozyme and target RNA. M, mutation; WT, wild-type sequence.*

patient, repair at the RNA level is less dangerous; even in the case of inadvertent changes to nontargeted RNAs, the effects would only be temporary.

Not less importantly, *trans*-splicing ribozymes and twin ribozymes are powerful tools in molecular biology and biochemistry. For example, twin ribozymes can be used for site-specific modification of long RNA molecules obtained by transcription. The repair oligonucleotide is synthetically available so a desired modification (for instance a fluorescence dye) can be introduced site specifically. The twin ribozyme accepts modified repair oligonucleotides for ligation[10c]. Thus, it should be possible to modify any transcript of unlimited length at a predetermined position, provided that this position is not buried in the secondary structure. This goal is difficult to achieve with current methods. So far the appropriate strategy would be chemical synthesis and modification of RNA fragments followed by enzymatic ligation, which is time consuming, rather expensive, and not very efficient.

**Keywords:** gene therapy · repair · ribozymes · RNA · sequence alteration

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